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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 9/96 — A2

(11) International Publication Number: WO 91/14773

(43) International Publication Date: 3 October 1991 (03.10.91)

(21) International Application Number: PCT/GB91/00443

(22) International Filing Date: 25 March 1991 (25.03.91)

(30) Priority data:
9006642.4 24 March 1990 (24.03.90) GB

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: ENZYME STABILISATION

(57) Abstract

A method of stabilising enzymes on drying and subsequent storage comprises the steps of mixing an aqueous solution of the enzyme with a soluble anionic polyelectrolyte and a cyclic polyol and removing water from the solution.

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ENZYME STABILISATION

This invention relates to the stabilisation of proteins, particularly, but not exclusively, of enzymes in the dry state.

PCT/GB89/01346 discloses stabilisation of proteins, particularly enzymes with combinations of cationic polyelectrolytes and cyclic polyols.

According to a first aspect of the present invention a method of protecting proteins against denaturation on drying comprises the steps of:

mixing an aqueous solution of the protein with a soluble anionic polyelectrolyte and a cyclic polyol, and removing water from the solution.

Biological activity in a protein, particularly activity in an enzyme system, may be enhanced upon drying with the stabilisers of this invention. The range of proteins includes enzymes, antibodies,, antigens, screen complement, vaccine components and bioreactive peptides. Freeze drying of samples may be employed. However, vacuum drying and air drying at ambient temperatures without denaturation is preferred.

In addition to retention of activity upon drying, proteins dried in the presence of the anionic polyelectrolyte and cyclic polyol may exhibit retention of activity after prolonged storage. Although various compounds have been used to provide active dried enzyme compositions, the present invention affords excellent characteristics or prolonged storage.

The cyclic polyol may incorporate one or more alicyclic rings and may have at least one side chain. Compounds having 5 to 10 hydroxyl groups may be preferred. Non reducing polyols are preferred. Disaccharides and derivatives are particularly efficaceous but other cyclic polyols, eg inositol, may be used. The polyol may be chosen to suit both the enzyme or other protein and also the polyelectrolyte in question.

Use of lactitol is especially preferred.

The amount of polyol may be in the preferred range 1 to 20%,

more preferably 2 to 10%. Percentages used in this specification are by weight to volume of aqueous solution unless indicated otherwise.

The anionic polymer is preferably a polymer with anionic groups distributed along the molecular chain. The anionic groups, which may include carboxyl, sulphonic acid, sulphate or other negatively charged ionisable groupings, may be disposed upon chain groups pendant from the chain or bonded directly to the polymer backbone.

Natural or artificial polymers may be employed. Natural polymers such as derivatised polysaccharides may be preferred since many synthetic polymers often contain residual traces of inorganic polymerisation catalysts. Alternatively synthetic polymers may be employed especially when used at very high dilution.

Carboxymethyl cellulose and sodium alginate (rich in galacturonic acid residues) are examples of carboxyl group containing polymers, dextran sulphate being an example of a sulphate containing polymer. Polymers with MW of 5,000-500,000 may be used, preferably 5,000 to 20,000 and more preferably 5,000 to 10,000. An amount of 0.05% to 10% w/v is preferred, especially 0.01 to 10%, more especially 0.5 to 2%. Use of a trace of the polyelectrolyte surprisingly affords excellent stabilisation, particularly on storage. Use of a minimal amount of the polyelectrolyte is preferred.

The pH at which the proteins may be dried in accordance with this invention may be important to optimum retention of activity both upon drying and after subsequent storage. The optimum pH for a particular enzyme may be determined by simple experimentation. Batches of enzymes from diffent sources have been found to require different conditions for optimum stabilisation.

Lactate dehydrogenase has been found to retain most activity between pH6.0 and pH7.0, especially at pH6.0.

Peroxidase retains most activity at pH7.0.

Alcohol oxidase is also stabilised by an anionic

polyelectrolyte/cyclic polyol combination at pH7.0.

Drying is preferably performed at temperatures between 4°C and 50°C, especially between 25°C to 35°C.

The dried product may be prepared as a free running powder or it may comprise part of a test strip or other analytic or diagnostic device or apparatus.

Use of the present invention finds particular application in stabilisation of proteins which have maximum activity at high pH, for example alkaline phosphatase. In addition the invention allows use of a particular enzyme in an assay system which employs alkaline reagents.

The present invention is now described by means of Examples but not in any limitative sense.

All stabilisation systems utilise buffers to maintain stable pH conditions.

A buffer solution containing $Na_2HPO_42H_2O$ (10.855g) and $NaH_2PO_42H_2O$ (6.084g) was dissolved in 1.0l distilled water to give a solution of pH7.0, at a concentration of 100mM/1. This was diluted as required.

An alternative buffer is MOPS (4-morpholino propane sulphonic acid) 52.25gm/2.5l distilled water to give a solution containing 100 millimoles per litre to which is added 4.0M NaOH to the required pH, eg pH7.

Wetting agents were not used in the following examples. However, this does not preclude their use in the stabilisation systems. Byco A, a protein (gelatin) hydrolysate may be used for example as a freshly made up solution of 1% concentration.

Enzyme solutions were prepared freshly before use. Stock suspensions of enzyme in ammonium sulphate solution were centrifuged and then redissolved and dialysed against a suitable buffer, or centrifuged, redissolved and used without further treatment.

Stock enzyme concentrations varied considerably, typically concentrations between 10 - 5,000 units of activity per millilitre of solution. The protein concentration being 0.05mg - $100mgcm^{-3}$.

The detection systems for each enzyme were those typically used in the published literature, Bergmeyer, H.U, Methods in Enzymatic Analysis, being the standard work employed.

The activity was measured kinetically at standard temperatures and the rate of reaction plotted automatically, using a Beckman Du-50 spectrophotometer fitted with a Kinetics Softpac Microprogram.

The dry preparations were produced by mixing the enzymes, buffers, anionic polyelectrolytes and polyols with stirring. Aliquots were dispersed into cuvettes and dried in a vacuum oven over silica gel as dessicant for 4 hours minimum at 30-35°C at 0.1mM mercury.

The stability of such preparations was tested by storage at elevated temperature, 37°C or 50°C being used. Samples were stored over silica gel as dessicant, removed periodically and reconsituted in the assay buffer recommended for the enzyme determination and checked for residual activity of the enzyme.

Example 1. Alcohol oxidase (Hansenula polymorpha. Prepared in house)

Alcohol oxidase 617 units cm^{-3} (Hansenula polymorpha)	16ul
Lactitol 20%	250ul
Sodium alginate 2%	250ul
Sodium phosphate buffer 10mM pH 7.0	484ul

The mixture was mixed and 0.1 ml volumes were dried in cuvettes as described, stored at 37°C and assayed for activity in a peroxidase dye linked reaction at 505 nM.

The results are shown in Table 1.

Example 2. L-lactate dehydrogenase (SIGMA Type II L 2500)

L-Lactate dehydrogenase (dialysed), approx 4,000 U cm^3	15ul
Sodium phosphate buffer pH 6.0 10mM	1110ul

These were mixed and added to 375ul of solutions of stabiliser as shown in Table 2.

The mixture was stirred well and 0.1 cm^{-3} placed in cuvettes which was vacuum dried as described, stored at 37°C and assayed by following the formation of Beta-NADH at 340 nm from L-lactate an Beta-NAD in glycine buffer at pH 8.9.

Example 3. L-lactate dehydrogenase (SIGMA Type II L-250)

L-lactate deydrogenase (dialysed) Approx 4,000 U cm^{-3}	15ul
Sodium phosphate buffer pH 6.0 10 mM	1110ul

These were mixed and added to 375 ul of a solution of stabilisers as shown in Table 3.

The mixtures was stirred well and 0.1cm^{-3} placed in cuvettes, dried as described, stored at 37°C and assayed by following the formation of Beta-NADH at 340 nm from L-lactate and Beta-NAD in glycine buffer at pH 8.9.

Example 4. Alkaline phosphatase (Sigm p-7640 Type 1-S)

Akaline phosphatase 6 ucm^{-3} (in pH 7.0 phosphate buffer 100 mM)	30ul
Distilled water	270ul
Sodium phosphate buffer pH 7.0 10mM	1200ul

These were mixed and added to 500ul of mixed solutions of stabiliser as shown in Table 4.

The mixtures were stirred well and 0.1 cm^{-3} placed in cuvettes and dried as described. The reactions were followed at 440 nM by the release of nitrophenol from the substrate 4-nitrophenol phosphate at pH 10.5 in 2 amino 2 methyl 1-propanol/HC1 buffer.

Example 5. Horseradish peroxidase (Sigma Type II) p-8250

Horseradish peroxidase 20 U cm^{-3}	300ul
Sodium phosphate buffer pH 7.0 10 mM	1200ul

These were mixed and added to 500ul of mixed solutions of stabilisers as shown in Table 5. Experiments were also carried out using alternative peroxidases.

The mixtures were stirred well and 0.1 cm^{-3} placed in cuvettes and dried as described. Activity was followed using the colorimetric reaction of 4 aminoantipyrine and phenolsulphonic acid with hydrogen peroxide as substrate measured at 505nM.

Example 6. Beta-Galactosidase (Sigma G-1875 Grade III)

Beta-Galacosidase 1 U cm^{-3}	25ul
(in 100mM pH 7.0 phosphate)	125ul
Sodium phosphate bufer pH 7.0 10mM	600ul

These were added to 250ul of a solution of stabilisers as shown in Table 6.

The mixture was stirred well, 0.1 cm^{-3} placed in a cuvette and dried as described. The activity was measured by following the release of O-nitrophenol at 405 nM from the substrate O-nitrophenyl-Beta-D-Galactopyranoside in maleate buffer at pH 7.3.

Example 7. Beta-Galactosidase (Sigma G-1875 Grade III)

Beta-Galactosidase 1 Ucm^{-3}	25ul
(in 100 mM pH 7.0 phosphate)	
Distilled water	125ul
Sodium phosphate buffer pH 7.0 10 mM	600ul

These were added to 250 ul of a mixed solution of stabilisers as shown in Table 7.

The mixture was stirred well and 0.1 cm^{-3} placed in cuvettes and dried as described. The activity was followed by the same procedure as before.

Example 8. Diacetyl reductase (Chicken liver) prepared in house

Diacetyl reductase 19 units cm^{-3}	100ul
Sodium phosphate buffer 10 mM pH 7.0	

These were added to 250ul of stabilisers as shown in Table 8.

The mixtures were stirred well and 0.1 ml volumes were dried in cuvettes as described, stored at 37°C and assayed for activity using the decrease of absorbance at 340 nM corresponding to the consuption of NADH in the reduction of diacetyl (2,3 butanedione) at pH 6.1.

Example 9. Lactate Dehydrogenase (Sigma Type II)

The dialysed (15ul of 4000 un/cm^{-3}) enzyme was added to 10mM sodium phosphate buffer 1110ul and 375ul of a solution of stabilisers was added to give the concentration shown in Table 9. The 100ul aliquots were dried as before. The activity was assayed using the same assay system as in Examples 2 and 3.

Example 10. Maleate Dehydrogenase

20ul of a suspension of malate dehydrogenase (Sigma 410-12 from porcine heart muscle) was centrifuged at 13500 rpm. The supernatant was discarded and the precipitate dissolved in 300ul 5mM phosphate buffer pH 6.0 and dialysed for two 2-hour periods against the same buffer.

150ul of dialysed enzyme was added to 250ul sodium phosphate buffer (pH 8, 100mM) and 250ul distilled water. 350ul of stabilisers was added to the mixture to give the concentrations shown in Table 10 and the 100ul alequots were dried at 35°C.

The enzyme activity was assayed in 100mM diethanolamine

buffer (pH 9.2) containing 5mM magnesium chloride and D,L-malate in excess (10 to 30mM). Beta-NAD (2.4mM) was added and the rate of production of Beta-NADH was measured by absorption at 340nm.

Table 1

Preparation	Incubation 37°C	% Activity remaining relative to the activity of fresh undried enzyme
Alcohol oxidase (Hansenula polymorpha	zero time	74
Lactitol 5%	1 day	88
Sodium alginate 0.5%	12 days	113
pH 7.0 phosphate	31 days	147
	48 days	118
	56 days	100

Unstabilised enzyme retained 25% activity after 1 day and 4% activity after 31 days at 37°C.

Table 2

Preparation	Incubation 37°C	% Activity remaining relative to the activity of fresh undried enzyme
L-lactate Dehydrogenase (Sigma type II)	zero time	96
Lactitol 3.5%	1 day	108
Dextran sulphate 0.71%	13 days	96
pH 6.0 phosphate	21 days	106
	30 days	93
	54 days	88

Unstabilised enzyme retained 55% activity after 1 day and 46% activity after 54 days at 37°C

Table 3

Preparation	Incubation 37°C	% Activity remaining relative to the activity of fresh undried enzyme
L-lactate dehydrogenase (Sigma type II)	zero time	-
Lactitol 2.75% Sodium Alginate 0.22% pH 6.0 phosphate	1 day	95
	13 days	89

Unstabilised enzyme retained 55% activity after 1 day and 46% activity after 54 days at 37°C.

Table 4

Preparation	Incubation 37°C	% Activity remaining relative to the density of final undried enzyme
Alkaline phosphase (Sigma type 1-5) pH 7.0 phosphate	zero time	100
a. Lactitol 3.5% Dextran sulphate 0.7%	1 day	89
	7 days	86
b. Lactitol 5% Dextran sulphate 0.71%	zero time	83
	15 days	95
c. Lactitol 2.7% Carboxymethyl cellulose 0.56%	zero time	100
	15 days	92

Unstabilised enzyme retained 80% activity after 1 day and 38% activity after 23 days at 37°C.

Table 5

Preparation	Incubation 50°C	% Activity remaining relative to the density of fresh undried enzyme
Horseradish peroxidase (Sigma type II) P8250	zero time	99
a. Lactitol 2.75%	1 day	69
Sodium carboxymethyl-cellulose 0.55%	7 days	79
pH 7.0 phosphate 10mM	15 days	79
	23 days	74
b. Lactitol 2.7%	zero time	102
Sodium carboxymethyl-cellulose 0.56%	15 days	73
c. Lactitol 2.7%	zero time	102
Dextran sulphate 0.71%	15 days	73
Horseradish peroxidase (Biozyme HRP-4b)	zero time	71
a. Lactitol 5%		
Dextran sulphate 1%	17 days	56
b. Lactitol		
Carboxymethyl cellulose	zero time	80
	17 days	35

Horseradish peroxidase (Biozyme HRP-5, 90% Isoenzyme C)			
a.	Lactitol 5%	zero time	87
	Dextran sulphate 1%	17 days	60
b.	Lactitol 5%	zero time	78
	Carboxymethyl cellulose 1%	17 days	65

Unstabilised enzyme retained 62% ativity after 1 day and 21% activity after 15 days at 50°C.

Table 6

Preparation	Incubation 50°C	% Activity remaining relative to the activity of fresh undried enzyme
Beta-Galacosidase (Sigma)	zero time	109
Lactitol 3.5%	1 day	91
Dextran sulphate 0.71%	7 days	86
pH 7.0 phosphate	10 days	87
	36 days	81

Unstabilised enzyme retained 66% activity after 7 days and 52% activity after 36 days at 50°C.

Table 7

Preparation	Incubation 50°C	% Activity remaining relative to the activity of fresh undried enzyme
Beta-Galactosidase (Sigma Type)	zero time	114
Lactitol 2.75%		
Sodium carboxymethyl cellulose	1 day	91
pH 7.0 phosphate 10mM	7 days	77
	10 days	89
	36 days	72

Unstabilised enzyme retained 66% activity after 7 days and 52% activity after 36 days at 50°C.

Table 8

Preparation	Incubation 37°C	% Activity remaining relative to the activity of fresh undried enzyme
Diacetyl reductase (Chicken liver)	zero time	216
pH 7.0 phosphate 10mM		
a. Lactitol 5%	8 days	144
Dextran sulphate 1%	21 days	134
b. Lactitol 5%	zero time	64
Carboxymethyl cellulose 1%	8 days	36
c. Lactitol 5%	zero time	68
Sodium alginate 0.5%	8 days	60

Unstabilised enzyme retained 9% activity ater 8 days at 37°C.

Table 9

Lactate dehydrogenase (Sigma Type II)		
Lactitol 5%	zero time	96
Dextran sulphate 0.7%	54 days	88

Unstabilised enzume retained 55% activity after 1 day and 46% activity after 54 days at 37°C.

Table 10

Maleate dehydrogenase (Sigma)		
Lactitol 5%	zero time	77
Carboxymethylcellulose 1%	20 days	89

Unstabilised enzyme retained 14% activity on drying, (aero time) and 2% activity after 20 days at 37°C

CLAIMS

1. A method of protecting proteins against denaturation on drying comprising the steps of:

mixing an aqueous solution of the protein with a soluble anionic polyelectrolyte and a cyclic polyol, and removing water from the solution.

2. A method as claimed in claim 1, wherein said polyelectrolytes comprise an anionic functionalised polysaccharide.

3. A method as claimed in claim 2 wherein the polyelectrolyte comprises dextran sulphate, laliginate or carboxymethylcellulose.

4. A method as claimed in any preceding claim wherein the polyol is a di- or trisaccharide.

5. A method as claimed in claim 4 wherein the polyol is selected from the group comprising lactitol, lactose, maltose, sucrose and cellobiose.

6. A method as claimed in any preceding claim wherein water is removed at a temperature of between 4° and 50°C.

7. A method as claimed in claim 6 wherein the temperature is 25° to 35°C.

8. A method as claimed in any preceding claim wherein the amount of anionic polyelectrolyte in the aqueous solution is from 0.005 to 10% w/v.

9. A method as claimed in claim 8 wherein the amount is 0.01 to 10%.

10. A method as claimed in claim 9 wherein the amount is 0.5 to 2%.

11. A method of protecting proteins against denaturation on storage comprising use of a method as claimed in any preceding claim.

12. A dried product containing a protein, cyclic polyol and an anionic polyelectrolyte.

13. A dried product as claimed in claim 12 containing an enzyme, cyclic polyol and an anionic functionalised polysaccharide.

14. A dried product as claimed in claim 13 containing an enzyme, lactitol and an anionic functionalised polymeric polysaccharide.

15. An enzyme assay system comprising a dried stabilised enzyme prepared in accordance with any of claims 1 to 11.